EFFECTS OF MALNUTRITION AND SUPPLEMENTED NUTRITION ON NONSPECIFIC IMMUNE FUNCTION CHANGES INDUCED BY FLUORIDE IN RABBITS

Bianhua Zhou,[a] Hongwei Wang, [a] Jinming Wang, [a] Jianhai Zhang,[a]
Xiaoyan Yan, [a] Jundong Wang, [a]
Shanxi, China

SUMMARY: This study was designed to investigate the protective role of protein (Pr) and calcium (Ca) on effects of fluoride (F) induced in nonspecific immunological function in New Zealand rabbits fed a Pr and a Ca nutritionally deficient (malnutrition) diet. Eighty healthy 30-day-old rabbits (1.07±0.25 kg) were divided randomly into four equal groups of twenty (female:male = 1:1). The four groups were maintained on distilled water and fed for 120 days: (1) a malnutrition control (MC) diet (8.58% Pr, 0.49% Ca = MC group); (2) the MC diet plus HiF (442 mg NaF (=200 mg F ion]/kg diet = HiF group); (3) a Ca deficient diet plus HiPr+HiF (18.41% Pr, 0.46% Ca, plus HiF diet = HiPr group); and (4) a Pr deficient diet plus HiCa+HiF (8.35% Pr, 2.23% Ca, plus HiF diet = HiCa group). Growth of the rabbits was markedly inhibited by HiF ingestion. Pr supplementation significantly alleviated the HiF-induced decrease in rate of growth. Compared to the MC group, the serum total Pr (TPr) and serum albumin (ALB) content in the HiF group were significantly decreased on average by 12.4% and 7.7%, respectively. Pr or Ca supplementation markedly increased serum TPr and ALB content compared to the HiF group. Compared to the MC group, tissue acid phosphatase (ACPase), serum lysozyme (Lys), and serum ACPase concentrations were significantly increased with HiF ingestion but were significantly alleviated by Pr and Ca supplementation. These findings indicate that HiF ingestion seriously damages nonspecific immune function in rabbits and that Pr and Ca can play protective roles against F-induced damage to immune functions.

Keywords: Dietary calcium; Dietary protein; High fluoride; Malnutrition; Nonspecific immunity; Rabbit immune function.

INTRODUCTION

Fluoride (F) is ubiquitous in varying amounts in food and water and is an air pollutant from numerous industrial operations. Exposure to F results in F absorption and transportation via the blood to tissues and organs causing structural changes and disturbance in their functions.[1] Thus, in addition to skeletal manifestations,[2-4] chronic F poisoning is known to cause a variety of pathological changes in non-skeletal tissues. Structural and functional changes in brain,[5-12] reproductive tissues,[13-18] thyroid gland,[19-25] collagen,[26-28] liver,[29] kidney,[30] and erythrocytes,[31] have been reported.

Epidemiological investigations have demonstrated that endemic fluorosis is mainly prevalent in undeveloped countries, particularly areas of malnutrition. Many studies report that protein (Pr) and calcium (Ca) malnutrition aggravates fluorosis,[32-35] and that protein and calcium supplementation can alleviate it.[33-35] Our previous studies indicate that high F and malnutrition aggravated fluorosis, and Pr supplementation had much better ameliorating effects on fluorosis in goats under pasture conditions.[2,3,36] However, some authors report that endemic

[a]For Correspondence: Prof Jundong Wang, Shanxi Key Laboratory of Ecological Animal Science and Environmental Medicine, Shanxi Agricultural University, Taigu, Shanxi, 030801, PR of China; E-mail: wangjd@sxau.edu.cn

fluorosis is also a Ca paradox disease, and that supplemental Ca has significant preventive effects.[35, 37]

The aim of this study was to investigate supplemented Pr and Ca on growth and general health in rabbits under artificially controlled conditions of Pr and Ca malnutrition and high fluoride exposure.

MATERIALS AND METHODS

Animals: Eighty healthy one-month-old healthy New Zealand rabbits (female:male = 1:1) weighing 1.07±0.25 kg) were obtained from the Rabbit Breeding Farm of Taigu Country and kept in a spacious animal house at 22–25ºC on a 12-hr light/dark cycle. All the animals were provided a similar diet of the same energy content *ad libitum* along with low-F distilled water. The study design was approved by the Institutional Animal Care and Use Committee of China.

Treatment: Details of the treatment of control and experimental groups are presented in Table 1. The rabbits were divided into four equal groups of twenty: group 1 was fed a Pr and Ca deficient, malnutrition control (MC) diet (8.58% protein, 0.49% calcium = MC group); group 2 was given the MC diet plus NaF (8.58% Pr, 0.49% Ca, plus 442 mg NaF/kg diet = HiF group); group 3 was given a Pr-rich, Ca-deficient diet plus HiF (18.41% Pr, 0.46% Ca, plus 442 mg NaF/kg diet = HiPr group); group 4 was given a Ca-rich plus the Pr-deficient diet and HiF (8.35% protein, 2.23% calcium, plus 442 mg NaF/kg = HiCa group). All the experimental rabbits were maintained on distilled water, and each rabbit was caged separately. The diets were prepared according to physical circumstances during the dry grass season (Table 1).

Table 1. F^- (mg/kg), Pr and Ca level (%,) and energy density (ED, as MJ/kg) in the diet of the rabbits

	Pr	Ca	P	F^-	ED
MC group	8.58	0.49	0.24	20.1	9.84
HiF group	8.58	0.49	0.24	200[a]	9.84
HiPr group	18.41	0.46	0.26	200[a]	10.37
HiCa group	8.35	2.23	1.33	200[a]	9.84

P denotes phosphorus. [a]From 442 mg/kg NaF. (A standard rabbit diet contains 12–16% protein and 1% Ca).

Biochemical examination: On the 30th, 60th, 90th, and 120th day of the feeding trial, eight rabbits were selected randomly from each group and deprived of food for 12 hr. After the rabbits were weighed, blood samples were collected by heart puncture. Serum was collected with centrifugation at 3000 rpm for 10 min and stored at –70ºC for analysis. Four rabbits in each group were euthanized by air injection in ear vein on the 30th, 60th, and 90th day, and eight on the 120th day. The spleen and thymus were immediately collected, weighed, and homogenized with 1:99 (w/v) and 1:9 (w/v) 0.9% saline solution at 4ºC, respectively. Serum TPr and ALB level, ACPase and lysozyme (Lys) activity, and tissue ACPase activity were determined with the reagent kit provided by the Nanjing Jianchen Biological Institute.

Pathology examination: On the 120th day, after the 8 rabbits were sacrificed, the spleen was removed for histological examination and fixed in 10%

formaldehyde solution, embedded in paraffin, serially sectioned at 5 micrometers, and stained with haematoxylin eosin.

Statistical analysis: Numerical results are expressed as mean±SD. Statistical analyses were performed by Student's t test. P<0.05 was considered as significant.

RESULTS

Data from the various treatments are summarized in Figures 1 and 2 and Tables 2–8. Figure 1 shows the growth curve of the rabbits.

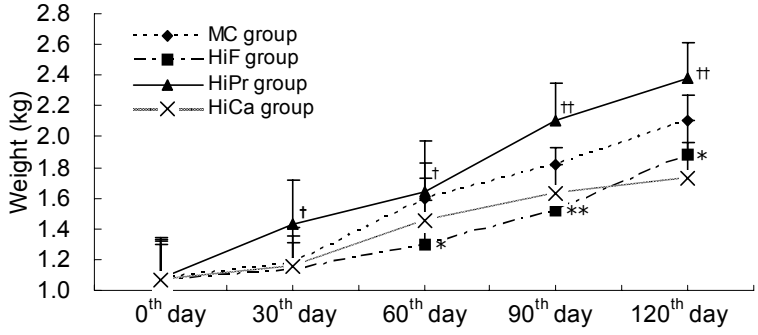

Figure 1. Increasing curve of weight in rabbits

*p<0.05, **p<0.01(HiF group compared with MC group); †p<0.05, ††p<0.01(HiPr group compared with HiF group, HiCa group compared with HiF group).

Tables 2–4 list the protein (Pr), calcium (Ca), and phosphorus (P) levels in the feces, and the serum total Pr and albumin, respectively.

Table 2. Pr, Ca, P levels (%) of the feces in rabbits (mean±SD; n=4)

	60th day			120th day		
	Pr	Ca	P	Pr	Ca	P
MC group	3.95±0.28	0.83±0.18	0.55±0.06	4.05±0.39	0.86±0.21	0.58±0.10
HiF group	4.44±0.28*	0.48±0.02**	0.29±0.04**	4.59±0.28	0.50±0.03*	0.29±0.04**
HiPr group	8.91±0.69††	0.79±0.09††	0.61±0.04††	9.07±0.76††	0.80±0.13††	0.62±0.04††
HiCa group	4.21±0.38	1.63±0.21††	0.90±0.14††	4.31±0.41	1.64±0.29††	0.91±0.18††

*p<0.05, **p<0.01(HiF group compared with MC group).
†p<0.05, ††p<0.01(HiPr group compared with HiF group; HiCa group compared with HiF group).

Table 3. Serum TPr level (mg/mL) in rabbits (mean±SD; n=8)

	30th day	60th day	90th day	120th day	Average
MC group	51.43±5.99	51.64±7.10	49.78±7.22	54.01±11.12	51.71±7.84
HiF group	48.41±7.43	46.42±11.74	44.43±8.09	41.95±10.24*	45.30±9.38**
HiPr group	57.12±4.91†	61.23±12.58†	54.99±11.25†	59.73±8.14††	58.27±9.52††
HiCa group	55.45±11.64	56.20±6.29	54.41±9.74†	54.88±9.01†	55.24±8.93††

*p<0.05, **p<0.01(HiF group compared with MC group).
†p<0.05, ††p<0.01(HiPr group compared with HiF group; HiCa group compared with HiF group).

Table 4. Serum ALB level (mg/mL) in rabbits (mean±SD; n=8)

	30th day	60th day	90th day	120th day	Average
MC group	30.05±3.61	31.62±2.52	28.73±4.45	31.21±2.31	30.40±3.37
HiF group	28.89±2.95	29.10±2.85	25.85±4.96	28.34±2.94*	28.05±3.61**
HiPr group	31.43±3.51	30.36±2.48	30.21±2.20†	31.72±2.82†	30.93±2.74††
HiCa group	29.68±3.76	31.95±3.47	27.56±3.46	29.07±2.43	29.56±3.54

*p<0.05, **p<0.01(HiF group compared with MC group).
†p<0.05, ††p<0.01(HiPr group compared with HiF group; HiCa group compared with HiF group).

Serum lysozyme (Lys) and acid phosphatase (ACPase) and tissue ACPase activities are listed in Tables 5–8, respectively.

Table 5. Serum Lys activity (U/mL) in rabbits (mean±SD; n=8)

	30th day	60th day	90th day	120th day	Average
MC group	534.3±115.8	355.8±61.7	297.7±110.1	501.1±40.9	422.2±130.3
HiF group	568.0±119.0	541.0±97.8**	500.0±87.0**	473.9±39.4	520.7±93.7**
HiPr group	477.0±99.3	455.6±97.7	502.3±86.9	409.9±61.7†	461.2±90.2†
HiCa group	444.4±104.3†	467.7±96.3	558.5±79.9	489.3±45.0	490.0±91.2

*p<0.05, **p<0.01(HiF group compared with MC group).
†p<0.05, ††p<0.01(HiPr group compared with HiF group; HiCa group compared with HiF group).

Table 6. Serum ACPase activity (U/100mL) in rabbits (mean±SD; n=8)

	30th day	60th day	90th day	120th day	Average
MC group	39.82±6.99	37.56±8.63	20.09±6.11	12.11±1.98	27.40±13.33
HiF group	52.31±9.84*	43.12±8.28	25.27±6.96	16.73±5.62*	34.36±16.12
HiPr group	54.47±9.94	48.15±8.74	23.72±5.00	17.72±3.39	36.01±17.30
HiCa group	40.67±10.87†	38.78±6.25	22.97±5.04	17.30±5.02	29.93±12.27

*p<0.05, **p<0.01(HiF group compared with MC group).
†p<0.05, ††p<0.01(HiPr group compared with HiF group; HiCa group compared with HiF group).

Table 7. Thymus ACPase activity (U/gprot) in rabbits (mean±SD)

	30th d (n=4)	60th d (n=4)	90th d (n=4)	120th d (n=8)	Average
MC group	83.95±3.16	110.83±10.56	100.04±13.17	42.06±10.77	75.79±31.06
HiF group	101.78±12.13*	127.53±10.61	119.88±13.78	51.18±13.44	90.31±35.86
HiPr group	80.95±13.03	87.35±18.21††	113.67±4.55	41.04±14.37	72.81±31.52
HiCa group	85.78±9.41	101.78±9.13†	98.48±17.46	46.94±12.62	75.98±27.50

*p<0.05, **p<0.01(HiF group compared with MC group).
†p<0.05, ††p<0.01(HiPr group compared with HiF group; HiCa group compared with HiF group).

Table 8. Spleen ACPase activity (U/gprot) in rabbits (mean±SD)

	30th d (n=4)	60th d (n=4)	90th d (n=4)	120th d (n=8)	Average
MC group	72.65±3.21	112.68±13.52	93.53±10.95	99.18±11.17	95.44±16.55
HiF group	96.91±9.06**	143.10±15.57*	133.08±15.61**	113.88±12.85*	120.17±20.66**
HiPr group	92.82±16.23	128.78±13.73	117.40±12.40	96.45±8.79††	106.38±18.32††
HiCa group	89.19±15.73	115.58±10.47†	98.01±8.07††	82.28±13.79††	93.47±17.33††

*p<0.05, **p<0.01(HiF group compared with MC group).
†p<0.05, ††p<0.01(HiPr group compared with HiF group; HiCa group compared with HiF group).

Morphological changes in spleen tissue of rabbits on the 120th day are shown in Figure 2.

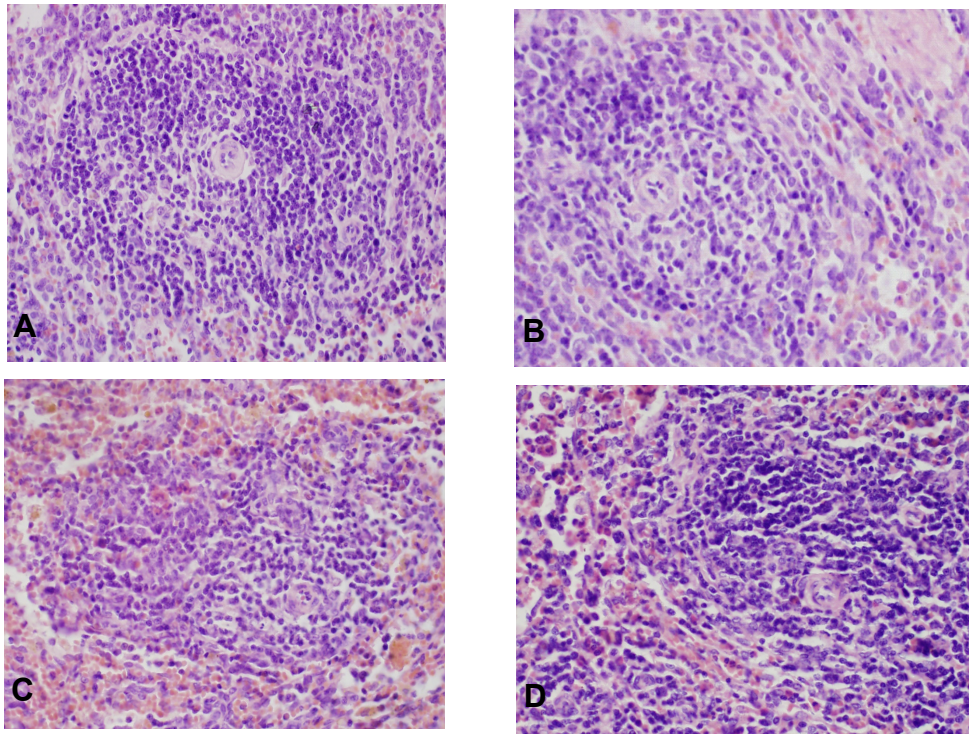

Figure 2. Morphological changes in spleen tissue of rabbits after 120 days stained with haematoxylin and eosin (HE). A, B, C, and D are sections of spleen tissue from the MC, HiF, HiPr, and HiCa groups, respectively (6.6×40).

As seen in Figure 2, in the MC group (A) the boundary between the cortex and medulla was clear, and considerable amounts of lymphocyte in white pulp and red pulp were observed. In the HiF group (B), rare lymphocytes were erratically arranged, and few lymph nodules were present. In the HiPr and HiCa groups (C and D), Pr and Ca supplementation played a protective role to some degree for F-induced pathological changes in the spleen.

DISCUSSION

Growth in the rabbits was significantly inhibited by high F, which is consistent with results of previous studies.[38-42] Pr and Ca supplementation markedly increased the body weight in the F-intoxicated rabbits, and 18.41% Pr supplementation was more effective than 2.23% Ca supplementation over the entire 120-day treatment period. Serum TPr and ALB contents were significantly decreased by 12.4% and 7.7% on average, respectively, in the HiF group. Previous studies in rats,[45] children,[46] rabbits,[44] and Tuj sheep,[1] support these findings. Pr supplementation significantly increased serum TPr and ALB contents by 28.6% and 10.3% on average, respectively. Moreover, Ca supplementation also markedly increased serum TPr contents by an average of 21.9%. The reduction in serum TPr and ALB contents in rabbits induced by F might be due to either increased proteolysis or decreased Pr synthesis.[43,44,49] It has been reported that F inhibits Pr

synthesis by weakening the beginning of the peptide chain and by preventing the production of peptide chains in ribosomes.[47,48] In this study, we observed that the P levels in 96-hr feces samples from the 57th to the 60th day were significantly greater by 12.4% in the HiF group. These results suggest that high F not only increased proteolysis or decreased Pr synthesis but also decreased intestinal absorption of Pr and ultimately reduced serum TPr and ALB levels and caused a lower weight gain of the rabbits.

Ca is widely believed to help alleviate F toxicity by forming insoluble CaF_2 in the intestines. Interestingly, we observed fecal Ca levels in the HiF group were significantly decreased by 42.2% on the 57th to the 60th day and by 41.9% on the 117th to the 120th day, compared with MC group. With the decrease of fecal Ca, the P levels were also significantly decreased in the feces by 47.3% and 50%, respectively. It is well known that Ca balance in the body is regulated by both intestinal and kidney function. During the experiment with the Ca-deficient diet, the feces calcium content was due mainly to inevitable skeletal loss of Ca to maintain body metabolism. High F inhibits cellular Ca efflux, resulting in an increase in Ca retention,[50,51] and more serious damage in case of Ca malnutrition.[35] Excessive ionized intracellular Ca may result in decreased fecal Ca. On the other hand, F also increases serum parathyroid hormone,[52] which promotes intestinal absorption of Ca and decreases fecal Ca.[50] Evidently the decrease in fecal P was tied to the fall in fecal Ca.

Nonspecific immunity plays an important defensive role against the encroachment of exotic matter. Chronic F poisoning is known to injure nonspecific immunity function,[38, 53-57] and ACPase is a marker enzyme for Lys, whose enzyme quantity and quality determine the immunological function of macrophages. In a study of F effects on the silkworm gut, Chen found an increase in ACPase activity.[58] In the present study, serum ACPase activities were significantly increased by 31.4% on the 30th day and 38.2% on the 120th day in the HiF group, compared with the MC group. The thymus ACPase activities were also markedly increased by 21.2% on the 30th day, and the spleen ACPase activities were significantly increased by 25.9% on average, in the HiF group. Lys is synthesized by macrophage and then rapidly released to the blood. In this study, we observed that serum Lys activity was markedly increased by 23.3% on average in HiF group, compared with MC group. These increases in enzyme activities can be seen as a consequence of disordered free radical metabolism induced by F. Excessive formation of free radicals and lipid peroxidation from fluoride damages tissue, cell membrane, inner structure, and the metabolism of biological molecules.[14,59-62] In this study, obvious pathological structural changes were observed in the spleen as seen in Figure 2 (B, C, and D). F-induced damage of macrophage and the spleen as an immune organ, finally caused an excessive release of ACPase and Lys. On the other hand, Lys, as an autophagosome, was also provoked by F, resulting in the marked activity increase of ACPase.[63]

Pr supplementation significantly decreased serum Lys activity by 11.4% on average. Likewise, the spleen and thymus ACPase were markedly decreased by

11.5% on average and by 31.5% on the 60th day, respectively. Ca supplementation also markedly decreased serum ACPase by 22.3% on the 30th day. Similarly, thymus and spleen ACPase were decreased by 20.2% on 60th day and 22.2% on average, respectively. The ameliorative effect of supplemented Pr or Ca may therefore be due to strengthening of physiological function and resistance.

In conclusion, these findings indicate that excessive F markedly inhibited growth and general health in rabbits and increased their nonspecific immune-related ACPase and Lys activities. Pr and Ca supplementation provided a protective role to some extent on growth and nonspecific immunologic functions induced by F. By comparison, protection by Pr supplementation was better than by Ca in these experiments.

ACKNOWLEDGEMENT

This research was sponsored by the China National Natural Science Foundation (Grant No. 30471303).

REFERENCES

1 Cenesiz S, Ozcan A, Kaya N, Baysu N, Karabulut AB. Chronic effects of fluoride in Tuj sheep on serum levels of total protein, albumin, uric acid, and nitric oxide and activities of lactate dehydrogenase and leucine aminopeptidase. Fluoride 2005;38(1):52-6.
2 Wang JD, Guo YH, Liang ZX, Hao JH. Amino acid composition and histopathology of goat teeth in an industrial fluoride polluted area. Fluoride 2003;36(3):177-84.
3 Wang JD, Hong JH, Li JP, Guo YH, Zhang JF, Hao JH. Effect of high fluoride and low protein on tooth matrix development in goats. Fluoride 2002;35(1):51-5.
4 Wang JD, Zhan CW, Chen YF, Li JX, Hong JP, Wang WF, et al. A study of damage to hard tissue of goats due to industrial fluoride pollution. Fluoride 1992;25(3):123-8.
5 Ge YM, Ning HM, Feng CP, Wang HW, Yan XY, Wang JD, et al. Apoptosis in brain cells of offspring rats exposed to high fluoride and low iodine. Fluoride 2006;39(3):173-8.
6 Ge YM, Ning HM, Wang SL, Wang JD. Effects of high fluoride and low iodine on brain histopathology in offspring rats. Fluoride 2005;38(2):127-32.
7 Ge YM, Ning HM, Wang SL, Wang JD. Comet assay of DNA damage in brain cells of adult rats exposed to high fluoride and low iodine. Fluoride 2005;38(3):209-14.
8 Wang JD, Ge YM, Ning HM, Wang SL. Effects of high fluoride and low iodine on biochemical indexes of the brain and learning-memory of offspring rats. Fluoride 2004;37(3):201-8.
9 Wang JD, Ge YM, Ning HM, Wang SL. Effects of high fluoride and low iodine on oxidative stress and antioxidant defense of the brain in offspring rats. Fluoride 2004;37(4):264-70.
10 Wu CX, Gu XL, Ge YM, Zhang JH, Wang JD. Effects of high fluoride and arsenic on brain biochemical indexes and learning-memory in rats. Fluoride 2006;39(4): 274-9.
11 Bhatnagar M, Rao P, Saxena A, Bhatnagar R, Meena P, Barbar S, et al. Biochemical changes in brain and other tissues of young adult female mice from fluoride in their drinking water. Fluoride 2006;39(4):280-4.
12 Chirumari K, Reddy PK. Dose-dependent effects of fluoride on neurochemical milieu in the hippocampus and neocortex of rat brain. Fluoride 2007;40(2):101-10
13 Zhang JH, Liang C, Ma JJ, Zhou BH, Wang JD. Changes in testis protein and metabolic enzyme activities in rats induced by sodium fluoride and sulfur dioxide. Fluoride 2006;39(3):179-84.
14 Zhang JH, Liang C, Ma JJ, Zhou BH, Wang JD. Effects of sodium fluoride and sulfur dioxide on oxidative stress and antioxidant defenses in rat testes. Fluoride 2006;39(3):185-90.
15 Wan SX, Zhang JH, Wang JD. Fluoride-induced changes in the expression of epidermal growth factor and its receptor in testicular tissues of young male rats. Fluoride 2006;39(2):121-5.

16 Zhang JH, Liang C, Ma JJ, Niu RY, Wang JD. Effects of sodium fluoride and sulfur dioxide on sperm motility and serum testosterone in male rats. Fluoride 2006;39(2):126-31.
17 Wan SX, Zhang JH, Wang JD. Effects of high fluoride on sperm quality and testicular histology in male rats. Fluoride 2006;39(1):17-21.
18 Pushpalatha T, Srinivas M, Reddy PS. Exposure to high fluoride concentration in drinking water will affect spermatogenesis and steroidogenesis in male albino rats. BioMetals 2005;18(3):207-12.
19 Ge YM, Ning HM, Wang SL, Wang JD. DNA damage in thyroid gland cells of rats exposed to long-term intake of high fluoride and low iodine. Fluoride 2005;38(4):318-23.
20 Susheela AK, Bhatnagar M, Vig K, Mondal NK. Excess fluoride ingestion and thyroid hormone derangements in children living in Delhi, India. Fluoride 2005;38(2):98-108.
21 Bouaziz H, Soussia L, Guermazi F, Zeghal N. Fluoride-induced thyroid proliferative changes and their reversal in female mice and their pups. Fluoride 2005;38(3):185-92.
22 Zhan XA, Li JX, Wang M, Xu ZR. Effects of fluoride on growth and thyroid function in young pigs. Fluoride 2006;39(2):95-100.
23 Schuld A. Is dental fluorosis caused by thyroid hormone disturbances?. Fluoride 2005;38(2):91-4.
24 Cinar A, Selcuk M. Effects of chronic fluorosis on thyroxine, triiodothyronine, and protein-bound iodine in cows. Fluoride 2005;38(1):65-8.
25 Bouaziz H, Ammar E, Ghorbel H, Ketata S, Jamoussi K, Zeghal N, et al. Effect of fluoride ingested by lactating mice on the thyroid function and bone maturation of their suckling pups. Fluoride 2004;37(2):133-42.
26 Yan XY, Li WT, Zhou BH, Wang JM, Wang JD. Effect of supplemented protein and Ca nutrition on fluoride-induced disturbance of rib COL1A1 gene expression in rabbits. Fluoride 2007;40(2):140-8.
27 Li WT, Yang LF, Ren YC, Yan XY, Wang JD. Quantification of rib COL1A2 gene expression in healthy and fluorosed Inner Mongolia cashmere goats. Fluoride 2007;40(1):13-8.
28 Li WT, Yang LF, Zhou BH, Yan XY, Wang JD. Effect of industrial fluoride pollution on COL2A1 gene expression in rib cartilage of Inner Mongolia cashmere goats. Fluoride 2006;39(4):285-92.
29 Shashi A. *In vivo* studies concerning toxic effects of sodium fluoride on hepatic function in rabbits. Fluoride 2003;36(1):30-7.
30 Zhan XA, Wang M, Xu ZR, Li JX. Toxic effects of fluoride on kidney function and histological structure in young pigs. Fluoride 2006;39(1):22-6.
31 Suska M, Janiak M. Relationship between cellular adenine nucleotide concentrations in erythrocytes and serum fluoride levels in two breeds of sheep. Fluoride 2006;39(1):60-4.
32 Kodali VRR, Krishnamachari KAVR, Gowrinathsastry J. Eruption of deciduous teeth: influence of undernutrition and environmental fluoride. Fluoride 1994;27(4): 236-7.
33 Chinoy NJ, Mehta D. Effects of protein supplementation and deficiency on fluoride-induced toxicity in reproductive organs of male mice. Fluoride 1999;32(4):204-14.
34 Chinoy NJ, Mehta D, Jhala DD. Effects of fluoride ingestion with protein deficient or protein enriched diets on sperm function of mice. Fluoride 2006;39(1):11-6.
35 Zhang WL, Zhao H, Xue LJ, Zhang XY, Li GS. Intracellular ionized calcium level and oxidative stress in kidneys of Wistar rats after intake of excessive fluoride. Chin J End. 2004;23(1):25-7. [in Chinese].
36 Wang JD, Hong JH, Li JX, Cai JP. The effect of nutrition supplementation during the annual dry grass season on tooth wear in industry-fluorosed goats. Fluoride 1994;27(3):136-40.
37 Zhang WL, Xing DL, Yang SJ, Li GS. Calcium and signal transmission in the activation of osteoblast in skeletal fluorosis. Chin J End. 2004;23(2):186-7. [in Chinese].
38 Vani ML, Reddy KP. Effects of fluoride accumulation on some enzymes of brain and gastrocnemius muscle of mice. Fluoride 2000;33(1):17-26.
39 Ramberg CF Jr., Phang JM, Mayer GP, Norberg AI, Kronfeld DS. Inhibition of calcium absorption and elevation of calcium removal rate from bone in fluoride-treated calves. J Nutr. 1970;100(8):981-9.
40 Cao J, Zhao Y, Liu JW, Shen N, Luo SF, Chen AC. The animal model of brick-tea-type fluorosis. Chin J End. 1998;17(5):287-90. [in Chinese].

41 Al-Hiyasat AS, Elbetieha AM, Darmani H. Reproductive toxic effects of ingestion of sodium fluoride in female rats. Fluoride 2000;33(2):79-84.
42 Shashi A. Histopathological investigation of fluoride-induced neurotoxicity in rabbits. Fluoride 2003;36(2):95-105.
43 Gogchaux W, Atwood KC. Structure and function of initiation complexes which accumulate during inhibition of protein synthesis by fluoride ion. J Bio Chem 1976;251(2):292-301.
44 Shashi A, Singh JP, Thapar SP. Protein degradation in skeletal muscle of rabbit during experimental fluorosis. Fluoride 1992;25(3):155-8.
45 Guan ZZ, Yang PS, Yu ND, Zhuang ZJ. An experimental study of blood biochemical diagnostic indices for chronic fluorosis. Fluoride 1989;22(3):112-8.
46 Shivashankara AR, Shankara YMS, Rao SH, Bhat PG. A clinical and biochemical study of chronic fluoride toxicity in children of Kheru Thanda of Gulbarga District, Karnataka, India. Fluoride 2000;33(2):66-73.
47 Chinoy NJ, Narayana MV, Sequeira E, Joshi SM, Barot JM, Purohit RM, et al. Studies on effects of fluoride in 36 villages of Mehsana District, North Gujarat. Fluoride 1992;25(3):101-10.
48 Michael M, Barot VV, Chinoy NJ. Investigations of soft tissue functions in fluorotic individuals of North Gujarat. Fluoride 1996;29(2):63-71.
49 Rzeuski R, Chlubek D, Machoy Z. Interactions between fluoride and biological free radical reactions. Fluoride 1998;31(1):43-5.
50 Das TK, Susheela AK. Effect of long-term administration of sodium fluoride on plasma calcium level in relation to intestinal absorption and urinary excretion in rabbits Environ Res 1993;62(1):14-8.
51 Borke JL, Whitford GM. Chronic fluoride ingestion decreases ^{45}Ca uptake by rat kidney membranes. J Nutr 1999;129(6):1209-13.
52 Yu J, Sun DJ, Wang W, Xu CB, Wu Y, Shi YX, et al. Changes of parathyroid hormone in the serum of fluorosis rats taking different levels of calcium. Chin J Endem 2006;25(6):625-8 [in Chinese].
53 Sjogren B. Fluoride exposure and respiratory symptoms in welders [abstract]. Fluoride 2005;38(4):329.
54 Steinegger S. Endemic skin lesions near an aluminum factory. Fluoride 1969;2(1): 37-9.
55 Litvinov NN, Goldberg MS, Kimina SN. Morbidity and mortality in man caused by pulmonary cancer and its relation to the pollution of the atmosphere in the areas of aluminum plants. Fluoride 1969;2(3):189-90.
56 Waldbott GL. Another fluoride fatality: a physician's dilemma [editorial]. Fluoride 1979;12(2):55-7.
57 Waldbott GL. Gastric ulcer and fluoride [editorial]. Fluoride 1977;10(4):149-51.
58 Chen YY, Du X, Jin YX. Cytochemical evidence for an anomalous dose-response of acid phosphatase activity in the blood but not the midgut of fluoride-treated silkworm larvae, *Bombyx mori*. Fluoride 2005;38(2):133-8.
59 Inkielewicz I, Rogowska M, Krechniak J. Lipid peroxidation and antioxidant enzyme activity in rats exposed to fluoride and ethanol. Fluoride 2006;39(1):53-9.
60 Zhan XA, Xu ZR, Li JX, Wang M. Effects of fluorosis on lipid peroxidation and antioxidant systems in young pigs. Fluoride 2005;38(2):157-61.
61 Güven A, Kaya N. Effect of fluoride intoxication on lipid peroxidation and reduced glutathione in Tuj sheep. Fluoride 2005;38(2):139-42.
62 Akdogan M, Eraslan G, Gultekin F, Sahindokuyucu F, Essiz D. Effects of fluoride on lipid peroxidation in rabbits. Fluoride 2004;37(3):185-9.
63 Li S, Kan DY, Guo TH, Xu SW, Kang SL. Effect of enzyme histochemistry on the non – skeletal tissues of chronic fluorosis in goat. J NE Agr Univ (China) 2006;37(3):345-8. [in Chinese].